Undiscovered role of endogenous thromboxane A_2 in activation of cardiac sympathetic afferents during ischaemia

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Myocardial ischaemia activates blood platelets, which in turn stimulate cardiac sympathetic afferents, leading to chest pain and sympathoexcitatory reflex cardiovascular responses. Previous studies have shown that activated platelets stimulate ischaemically sensitive cardiac sympathetic afferents, and that thromboxane A_2 (TxA_2) is one of the mediators released from activated platelets during myocardial ischaemia. The present study tested the hypothesis that endogenous TxA_2 stimulates cardiac afferents during ischaemia through direct activation of TxA_2 (TP) receptors coupled with the phospholipase C–protein kinase C (PLC–PKC) cellular pathway. Nerve activity of single unit cardiac sympathetic afferents was recorded from the left sympathetic chain or rami communicantes (T_2–T_5) in anaesthetized cats. Single fields of 39 afferents (conduction velocity = 0.27–3.65 m s^{-1}) were identified in the left or right ventricle initially with mechanical stimulation and confirmed with a stimulating electrode. Five minutes of myocardial ischaemia stimulated all 39 cardiac afferents (8 Aδ-, 31 C-fibres) and the responses of these 39 afferents to chemical stimuli were further studied in the following four protocols. In the first protocol, 2.5, 5 and 10 μg of the TxA_2 mimetic, U46619, injected into the left atrium (LA), stimulated seven ischaemically sensitive cardiac afferents in a dose-dependent manner. Second, BM13,177, a selective TxA_2 receptor antagonist, abolished the responses of six afferents to 5 μg of U46619 injected into the left atrium and attenuated the ischaemia-related increase in activity of seven other afferents by 44%. In contrast, cardiac afferents, in the absence of TP receptor blockade responded consistently to repeated administration of U46619 ($n = 6$) and to recurrent myocardial ischaemia ($n = 7$). In the fourth protocol, administration of PKC-(19–36), a selective PKC inhibitor, attenuated the responses of six other cardiac afferents to U46619 by 38%. Finally, using an immunohistochemical staining approach, we observed that TP receptors were expressed in cardiac sensory neurons in thoracic dorsal root ganglia. Taken together, these data indicate that endogenous TxA_2 contributes to the activation of cardiac afferents during myocardial ischaemia through direct stimulation of TP receptors probably located in the cardiac sensory nervous system and that the stimulating effect of TxA_2 on cardiac afferents is dependent, at least in part, upon the PLC–PKC cellular pathway.

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Activation of cardiac spinal (sympathetic) afferents during myocardial ischaemia elicits the perception of cardiac pain and initiates excitatory cardiovascular reflexes (White, 1957; Malliani, 1990; Meller & Gebhart, 1992). Although previous studies have investigated the central and peripheral neural mechanisms of cardiac–cardiovascular reflexes and cardiac pain (Foreman, 1999; Qin *et al.* 2003), we are just beginning to investigate the peripheral sensory signalling mechanisms underlying activation and sensitization of cardiac spinal afferents during myocardial ischaemia. We and others have demonstrated that a number of ischaemic metabolites, including histamine, 5-hydroxytryptamine (5-HT), lactic acid (protons), prostaglandins, reactive oxygen species and bradykinin (BK), in an interactive and multifactorial fashion, stimulate cardiac spinal afferents during ischaemia and

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reperfusion (Uchida & Murao, 1974; Baker *et al.* 1980; Malliani *et al.* 1981; Pan *et al.* 1999; Tjen-A-Looi *et al.* 2002; Fu & Longhurst, 2002*a*, 2005; Fu *et al.* 2005).

TxA_2 is produced during myocardial ischaemia and functions as a potent vasoconstrictor and platelet aggregator (Hirsh *et al.* 1981; Parratt & Cokerm, 1981; Fitzgerald *et al.* 1986; Arita *et al.* 1989). For instance, TxA_2 is produced in large quantities in the coronary circulation of patients with coronary artery disease (Mehta *et al.* 1984*a*). Occlusion of the coronary artery in animals also results in an increase in TxA_2 concentration (Hirsh *et al.* 1981). However, the role of TxA_2 with respect to stimulation of the sensory nervous system remains poorly investigated. Only recently have investigators begun to identify the actions of TxA_2 on neuronal activity. In this respect, previous studies have shown that the stable TxA_2 analogue U46619 stimulates 45% of group III and IV somatic sensory nerve fibres (Kenagy *et al.* 1997). U46619 is also capable of stimulating cardiac vagal afferent nerves that produce reflex bradycardia (Wacker *et al.* 2002). None of these studies examined the role of endogenous TxA_2. However, they did lead us to speculate that endogenous TxA_2 may play an important role in activating cardiac sympathetic nerve endings during myocardial ischaemia.

TxA_2 receptors or TxA_2/prostaglandin H_2 (PGH_2) receptors (termed TP receptors) have been demonstrated to be located on platelets and smooth muscle cells. Both TxA_2 and PGH_2 bind to TP receptors, leading to platelet aggregation and smooth muscle contraction (Coleman *et al.* 1994). In contrast, only a few recent studies have suggested that TP receptors exist on neurons and structural elements in the central and peripheral nervous systems. In this respect, immunohistochemical studies have revealed the presence of TP receptors on oligodendrocytes and astrocytes associated with myelinated fibre tracts, most notably in the striatum, spinal cord, and optic tract (Borg *et al.* 1994; Blackman *et al.* 1998). TxA_2 receptors also have been demonstrated to be located on Schwann cells of sciatic nerves and nodose ganglion neurons in rats (Muja *et al.* 2001; Wacker *et al.* 2005). We therefore speculated that TP receptors exist on cardiac sensory nerves.

The action of TxA_2 is mediated by the TP receptor, which is coupled to a G protein receptor. Specifically, the TP receptor is linked to Gq-dependent activation of phospholipase C (PLC) that, in turn, cleaves target membrane lipids to produce phosphoinositide (PI) to mobilize intracellular Ca^{2+} and diacylglycerol (DAG) to activate protein kinase C (PKC) (Bevan, 1996). TxA_2 alters neural transmission in the hippocampus through a PKC mechanism (Hsu & Han, 1996). We have observed that histamine and BK activate sympathetic visceral afferents during ischaemia, partially through a PKC mechanism (Guo *et al.* 1998; Guo & Symons & Longhurst, 1999; Fu *et al.* 2005). Together, these studies suggest that the PLC–PKC pathway plays a role in TxA_2-mediated activation of ischaemically sensitive cardiac afferents.

The aim of the present study was to determine if endogenously produced TxA_2 contributes to activation of cardiac sympathetic afferents during myocardial ischaemia though the TP–PKC intracellular pathway. We hypothesized that TxA_2 stimulates cardiac spinal afferents during myocardial ischaemia through a direct action on TP receptors, located on cardiac sensory nerves, and that the action of TxA_2 on cardiac afferents is dependent, at least in part, upon a PLC–PKC cellular pathway. A preliminary report of part of this study has been published (Fu & Longhurst, 2002*c*).

Methods

Surgical preparation

Adult cats of either sex (2.9 ± 0.28 kg, mean ± S.E.M.) were anaesthetized by intramuscular injection of ketamine (20–30 mg kg^{-1}, Phoenix Scientific, Inc., St Joseph, MO, USA), followed by intravenous injection of α-chloralose (40–50 mg kg^{-1}) through the femoral vein. Additional injections of α-chloralose (5–10 mg kg^{-1}, I.V.) were given as necessary to maintain an adequate depth of anaesthesia that was assessed by observing the absence of a conjunctival reflex. The trachea of each animal was intubated and respiration was maintained artificially (Harvard pump, model 661, Ealing, South Natick, MA, USA). Cats were ventilated by air supplemented with 100% O_2 through the respirator. The femoral vein and artery were cannulated for administration of drugs and fluid, and the measurement of blood pressure, respectively. Arterial blood pressure was measured by a pressure transducer (Statham P23 ID, Gould) connected to the femoral arterial catheter. Arterial blood gases were assessed frequently by a blood gas analyser (Radiometer ABL-5, Copenhagen, Denmark) and maintained within physiological limits ($P_{O_2} > 100$ mmHg, $P_{CO_2} = 28$–35 mmHg, pH 7.35–7.45) by adjusting the respirator rate or tidal volume, or by intravenously administering 2–3 ml of 1 M $NaHCO_3$ (8.4%, w/v). Another PE 90 catheter was introduced into the left atrium through the left atrial appendage for intracardiac injection of solutions. Body temperature was monitored by a rectal thermistor and maintained at 36–38°C with a circulating water heating pad and a heat lamp. At the end of the experiment, animals were killed by administration of a solution of saturated potassium chloride into the femoral vein under deep anaesthesia, ensured by administering an additional dose of α-chloralose (50 mg kg^{-1}). Surgical and experimental protocols used in this study were approved by the Animal Use and Care Committee at the University of California at Irvine. The studies conformed to the American

Physiological Society's 'Guiding Principles in the Care and Use of Animals'.

Cardiac spinal afferent recording

Single-unit activity of cardiac afferents was recorded as previously described (Fu & Longhurst, 2002*b*, 2005). In brief, a midline sternotomy was performed and the first to seventh left ribs and the left lung were removed. The left paravertebral sympathetic chain was isolated, then draped over a Plexiglas platform and covered with warm mineral oil. Small nerve filaments were dissected gently from the chain and rami communicantes between T_2 and T_5 under an operating microscope (Zeiss, Germany) and the rostral ends were placed across one pole of the recording electrode. The other pole of the recording electrode was earthed with a cotton thread to the animal. The recording electrode was attached to a high impedance probe (model HIP511, Grass Instruments, Quincy, MA, USA). Action potentials of afferents were amplified ($\times$50 000) and band-pass filtered (100–3000 Hz) through an AC amplifier (model P511 Preamplifier, Grass) and processed through an audioamplifier (AM8B, Audiomonitor, Grass) and an oscilloscope (model 2201, Tektronix, Beavertown, OR, USA). Nerve activity and blood pressure signals were recorded on a Pentium computer using data acquisition and analysis software (Spike2), which sampled these signals at 10 000 Hz through an analog-to-digital converter (CED micro 1401 mkII, Cambridge, UK) for on- and offline quantitative analysis. Discharge frequency was quantified by using a software window discriminator; a histogram was generated for each afferent. Accurate counting of the impulse activity of each afferent was verified by comparing the constructed histogram with the original neurogram.

The receptive field of each afferent was located by mechanical stimulation of the heart. This included constricting the thoracic aorta as well as gently probing the heart with a cotton swab. The location of the afferent nerve ending was confirmed further by placing a stimulating electrode directly on the surface of the myocardium to evoke the afferent's action potential. Briefly, while recording afferent fibre activity the epicardium was mapped gradually from the apex to the base of the heart using a bipolar stimulating electrode to search for the location of nerve endings electrically (6–12 V, 0.5 ms, 1 Hz). Once an action potential was evoked, the precise location of the receptive field was measured by gradually moving the stimulating electrode around the initially identified spot using a minimal intensity of stimulation. The conduction velocity of each afferent fibre was calculated by dividing conduction distance by conduction time. The conduction time was determined by measuring the time interval from electrical stimulation to the evoked afferent's action potential. The conduction distance was estimated by measuring the length of a wet thread between the receptive field and the recording electrode (Fu & Longhurst, 2002*b*, 2005). Unmyelinated C- and finely myelinated Aδ-fibre afferents were classified as those with conduction velocities (CVs) of < 2.5 and 2.5–30 m s^{-1}, respectively. In the present study, each afferent had a single receptive field that could be located precisely in the ventricles. Myocardial ischaemia was induced by complete occlusion of the appropriate coronary artery supplying the regional receptive field of the cardiac afferent nerve with a thread placed around the vessel. Ischaemia was confirmed by observing a regional change in the colour of the myocardium, which has been closely correlated to the production of lactic acid as indicated by a reduction in tissue pH (Pan *et al.* 1999). Afferents were considered to be ischaemically sensitive if their discharge activity during 3–5 min of myocardial ischaemia increased at least twofold above baseline activity (Fu & Longhurst, 2002*b*, 2005).

Surgical preparation for cardiac sensory neuron (CSN) labelling

Pre-anaesthesia of cats was induced with subcutaneous ketamine and midazolam at a dose of 5–10/0.1–0.2 mg kg^{-1}. Cats were intubated with a cuffed endotracheal tube and connected to an anaesthesia machine (model: NARKOMED II, North American Drager, Telford, PA, USA). Anaesthesia was maintained with isoflurane (1–2%) in 100% oxygen mixed with room air through inhalation. Similar to the afferent recording studies, during the surgical procedures for labelling cardiac sensory nerves, body temperature, monitored with a rectal probe, was maintained at 37°C with a circulating heating pad and a heat lamp. Blood oxygenation and heart rate were monitored using a pulse oximeter (model: 8500AV, Nonin Medical, Plymouth, MN, USA). The heart was exposed through a left lateral thoracotomy at the fifth intercostal space. One hundred microlitres of a suspension of 17 mg ml^{-1} of 1,1′-dioctadecyl-3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI; Molecular Probes) in a solution of saline was injected into the pericardial space to access the ventricular wall of the heart. Care was taken to eliminate any leaks of DiI around the injection site by tightening the small puncture hole with a silk suture. We observed no leakage over a 5 min period after injection. Next, the ribs were approximated, the thoracic cavity was evacuated and the incision was closed in layers. Post-operatively the cats were isolated and treated for pain and infection. Pain was controlled by prophylactic administration of buprenorphine (0.01 mg kg^{-1}, I.M.) every 8–12 h for the first 24–48 h period while infection was prevented by administration of penicillin G procaine (20 000 IU kg^{-1}, I.M.) every 8–12 h for 4 days.

Experimental protocols

Dose–responses of ischaemically sensitive cardiac spinal afferents to U46619. This protocol examined the response of ischaemically sensitive cardiac spinal afferents ($n = 7$ afferents) to graded doses of U46619 (2.5, 5 and 10 μg). After identifying the location of the receptive field of an afferent fibre in the ventricles, the response of the cardiac afferent was measured during 3–5 min of myocardial ischaemia. If the afferent responded to ischaemia, then U46619 or 2% ethanol (vehicle) was injected into left atrium (LA) and the afferent activity was recorded. Dose–response curves were generated with three different doses of U46619 (2.5, 5 and 10 μg, Sigma-Aldrich, St Louis, MO, USA) applied at least 20 min apart to avoid tachyphylaxis. U46619 and the vehicle were applied randomly. To prepare a stock solution, two milligrams of U46619 were dissolved in 0.4 ml of 100% ethanol to achieve an initial concentration of 5 mg ml^{-1} that was stored in a -70°C freezer. A working solution of 100 μg ml^{-1} was made by first removing 20 μl from the U46619 stock solution and adding 980 μl of 0.9% saline to obtain the final concentration. Solutions of 25 and 50 μg ml^{-1} of U46619 were made by further diluting the 100 μg ml^{-1} solution with 0.9% saline. Ethanol (2%) served as the vehicle.

Effect of TP receptor blockade on responses of U46619. In this protocol, we examined the influence of blockade of TP receptors with BM13,177 on afferent responses to U46619. After locating the receptive field of an afferent on the heart, the response to brief myocardial ischaemia was measured. If the afferent responded to ischaemia, we recorded the response to LA injection of U46619 (5 μg). This dose of U46619 was chosen based on the U46619 dose–response data. Repeated LA injections of U46619 were conducted 15 min after the intravenous administration of BM13,177 (30 mg kg^{-1}, Hoffmann-La Roche, Nutley, NJ, USA) and 30 min after the initial stimulation with U46619. BM 13,177 was dissolved in 1 ml of 8.4% $NaHCO_3$ and diluted as needed with 0.9% saline to a concentration of 30 mg ml^{-1}. Previous studies have demonstrated that this dose of BM13,177 selectively and completely inhibits platelet aggregation by blocking TxA_2 receptors (Nossaman *et al.* 1992; oude Egbrink *et al.* 1993). We administered BK (3 μg) into the LA to establish the responsiveness of the afferent after treatment with BM13,177. A total of six ischaemically sensitive afferents were studied in this group.

To determine the reproducibility of afferent responses to U46619, six additional afferents in six animals were studied as time controls. After identifying an ischaemically sensitive unit, each animal in this group was treated identically, except that vehicle (2% $NaHCO_3$, 3–5 ml, I.V.) was used in place of BM13,177.

Effect of TP receptor blockade on responses of afferents to ischaemia. This protocol consisted of two groups of afferents to examine the response of cardiac afferents to myocardial ischaemia before and after blockade of TP receptors with BM13,177. Seven afferents were studied in this group. After locating the receptive field of an afferent on the heart, discharge activity was measured during 5 min of regional myocardial ischaemia. If the afferent responded to ischaemia, a second period of ischaemia was repeated 30–40 min later in the presence of BM13,177 (30 mg kg^{-1}, I.V.). We administered BK (3 μg) into the LA to establish the responsiveness of the afferent 20 min after the second period of ischaemia.

To differentiate between variations in afferent responses related to drug effect and time-related effects, seven additional ischaemically sensitive afferents were examined to determine the repeatability of the afferent response to ischaemia in another group of seven animals. In this group, after identification, each afferent fibre was treated in an identical manner, except that the vehicle (2% $NaHCO_3$) was used in place of BM13,177.

Effect of PKC-(19–36) on response of afferents to U46619. In this protocol, we evaluated the effect of PKC-(19–36) (30 μg kg^{-1}, I.V.) on the response of six ischaemically sensitive afferents to U46619. After identification of an ischaemically sensitive unit, we injected U46619 (5 μg) into the left atrium while recording afferent activity. We repeated the application of U46619 (5 μg, LA) 15–20 min after its initial injection, including at least 15 min following treatment with PKC-(19–36). PKC-(19–36) is a highly selective inhibitor of PKC (Taiwo & Levine, 1991; Ahlgren & Levine, 1994). PKC-(19–36) (Sigma-RBI) was dissolved in distilled water to a concentration of 300 μg ml^{-1} and was further diluted to a final concentration using 0.9% NaCl. This dose of PKC-(19–36) has been shown previously to reduce the response of ischaemically sensitive cardiac afferents to histamine (Fu *et al.* 2005). After treatment with PKC-(19–36), we administered BK (3 μg) into the LA to establish the responsiveness of the afferent nerve ending. The above-noted group studied during repeat U46619 served as the time control for this protocol.

Histochemistry and immunohistochemistry to identify labelled CSNs. *Dorsal root ganglia (DRG) tissue preparations.* During the 10 week post-operative period, DiI was carried by retrograde transport back to the cell bodies of the CSNs. The cats were then killed. In brief, cats were re-anaesthetized with ketamine (40–50 mg kg^{-1}, I.M.). A femoral vein was cannulated for administration of drugs and fluids. A large dose of α-chloralose (100 mg kg^{-1}, I.V.) was administered to induce deep anaesthesia as judged by the lack of a withdrawal response to toe pinch and corneal reflexes. Subsequently, animals

were perfused transcardially with 0.9% saline and cold 4% paraformaldehyde in phosphate buffer (PB, pH 7.2). The DRGs at levels of L_1 (as control) and $T_{1–3}$ were harvested bilaterally since they are associated with cardiac sympathetic sensory pathways (Kuo *et al.* 1984). These DRGs were stored in 4% paraformaldehyde for 2 h, then transferred to 30% sucrose for 48 h to prevent ice crystallization.

Transverse 30 μm DRG sections were cut with a cryostat microtome (Leica CM1850 Heidelberger Strasse, Nussloch, Germany) and mounted serially on super-frost/plus slides (Fisher scientific, Pittsburgh, PA, USA). The sections were used to detect DiI staining and to conduct immunohistochemical labelling for the TP receptor, a 7-transmembrane-domain G-protein-coupled receptor.

Fluorescent immunohistochemical labelling for TP receptors. After rinsing three times (10 min each) with phosphate-buffered saline containing 0.3% Triton X-100 (PBST, pH = 7.4), DRG sections were treated with 1% normal donkey serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 1 h to block non-specific binding. The DRG sections were incubated with a rabbit anti-TP receptor polyclonal antibody (3.6 μg ml^{-1}, Cayman Chemical, Ann Arbor, MI, USA) in PBST at 4°C for 48 h. This antibody was raised specifically against TP receptor C-terminal amino acids 323–343 of human. The sections were then incubated with fluorescein-conjugated donkey anti-rabbit antibodies (1 : 100; Jackson Immunoresearch Laboratories, Inc.) in PBST for 24 h at 4°C. After rinsing the sections in phosphate-buffered saline (PBS, pH = 7.4) for 30 min (10 min × 3), they were air dried. The slides were coverslipped using mounting medium (Vector Laboratories, Burlingame, CA, USA). Immuno-histochemical control studies were performed by omission of the primary or secondary antibodies. No labelling was detected under these conditions.

DRG sections were examined with a standard fluorescence microscope (Nikon, E400, Melville, NY, USA). Two epifluorescence filters (B-2A or G-2A), equipped in a fluorescence microscope, were used to identify single stains appearing as green (fluorescein), or red (DiI) in DRG sections. Selected sections were evaluated with a laser-scanning confocal microscope (Zeiss LSM 510, Meta system, Thornwood, NY, USA) to confirm co-localization of two labels in the same cell. This apparatus is equipped with Argon and HeNe lasers to allow the operation of multiple channels. Lasers of 488 and 543 nm wavelengths were used to excite fluorescein (green) and DiI (red), respectively. Digital images of the immunoreactive structures were captured and analysed with software (Zeiss LSM) provided with the confocal microscope. Images in two colours in the same plane were merged to reveal the relationship between two immunoreactive elements. We identified cells that contained one-third or more of the cytoplasm stained bright red as DiI positive. We categorized a cell as TP receptor positive if it contained diffuse labelling with bright green fluorescence. Co-localization of two labels was identified by the appearance of yellow and/or orange, reflecting a mixture of red and green.

Data analysis

The discharge activity of cardiac spinal afferents was expressed in impulses per second and was averaged during the 3–5 min of pre-ischaemia and the 5 min of ischaemia. We measured the responses of cardiac afferent nerve endings to U46619, BM13,177 and PKC-(19–36) by averaging the discharge rates of the afferents during the entire period of the response, defined as the time during which sustained activity exceeded baseline activity by 20%. Five minute sampling periods were used to measure afferent activity during myocardial ischaemia. During drug injection, sampling periods varied between 1.5 and 8 min, depending on the responses of the afferent to the drug. Baseline activity was determined over the 3–5 min period immediately preceding ischaemia.

Data are expressed as means ± S.E.M. The effects of repeated injection of U46619, BM13,177, PKC-(19–36), and repeat ischaemia on the responses of the afferents were compared using a one-way repeated-measures analysis of variance (ANOVA) followed by Tukey's *post hoc* test. If the data were not normally distributed, as determined by the Kolmogorov–Smirnov test, they were compared with the Friedman repeated-measures analysis of variance on ranks and a Dunnett's *post hoc* test. We compared the effect of U46619 on the afferent discharge activity using Student's paired *t* test. Alternatively, if data were not normally distributed, we used the Wilcoxon signed rank test to compare the paired data. All statistical calculations were performed with SigmaStat software (Jandel Scientific Software, San Rafael, CA, USA). Values were considered to be significantly different when $P < 0.05$.

Results

Profile of cardiac afferents

The activities of 39 ischaemically sensitive cardiac afferents were recorded in the present study. Approximately 25% of these afferents were mechanosensitive, while all were chemosensitive. Endings of most (92%) afferents were located in the anterior ($n = 15$) and posterior ($n = 21$) wall of the left ventricle (Fig. 1). Three afferents were located on the posterior wall of the right ventricle. The conduction velocity for these afferents ranged from 0.27 to 3.65 m s^{-1}. Seventy-nine per cent (31 fibres) of these afferents

were classified as C-fibres ($CV = 0.74 \pm 0.12$ m s^{-1}). The remaining units (8 afferents) were classified as Aδ-fibres ($CV = 3.08 \pm 0.17$ m s^{-1}). No obvious association was found between conduction velocity and the responsiveness of the fibres to chemical stimulation or ischaemia.

Effect of U46619 on activity of ischaemically sensitive afferents

Injection of U46619, a TxA_2 mimetic, stimulated all ischaemically sensitive cardiac sympathetic afferents tested. Figure 2 displays nerve activity responses of an ischaemically sensitive cardiac C-fibre ($CV = 0.42$ m s^{-1}) during injection of vehicle (Fig. 2*A*) and U46619 (2.5–10 μg, Fig. 2*B–D*) into the left atrium. Discharge activity of this cardiac C-fibre afferent increased from 0.78 to 2.35 impulses s^{-1} during ischaemia. After release of coronary arterial occlusion, discharge activity of this fibre gradually returned to control levels. Injection of increasing doses of U46619 into left atrium led to a graded increase in afferent activity. In contrast, injection of vehicle (Fig. 2*A*) did not alter impulse activity. This afferent innervated the posterior wall of the left ventricle.

The effects of U46619 on the entire group of seven ischaemically sensitive cardiac afferents (two Aδ-, $CV = 2.75$ and 3.6 m s^{-1}; five C-fibres, $CV = 0.74 \pm 0.25$ m s^{-1}) are summarized in Fig. 3. In our preliminary study we observed that LA injection of 1.25 μg of U46619 only stimulated 2 of the 4 afferents that were studied. However, higher doses of U46619 (2.5–10 μg, LA) stimulated all seven fibres, significantly increasing their discharge activity in a dose-dependent manner (Fig. 3), with responses ranging from 0.72 ± 0.10 to 1.35 ± 0.18, 1.91 ± 0.33 and 2.44 ± 0.31 impulses s^{-1}. In contrast, injection of the vehicle did not stimulate any of the six fibres tested (0.62 ± 0.10 to 0.67 ± 0.11 impulses s^{-1}). The locations of each of the seven afferent nerve endings that responded to the TxA_2 mimetic are shown in Fig. 1.

Effect of blockade of TxA_2 receptors on cardiac afferents' responses to U46619

The responses of two groups of cardiac afferents to U46619 are displayed in Fig. 4. In the first group, U46619 (5 μg) significantly increased the discharge activity of six afferents (one Aδ-, $CV = 3.22$ m s^{-1}; five C-fibres, $CV = 0.59 \pm 0.28$ m s^{-1}) from 0.84 ± 0.20 to 2.38 ± 0.49 impulses s^{-1} (Fig. 4*A*). After blockade of TP receptors with BM13,177, however, the responses of these afferents to U46619 were abolished. BM13,177 itself did not stimulate or inhibit these afferents. In addition, after blockade with BM13,177, these afferents still responded to application of BK (0.79 ± 0.23 to 3.38 ± 0.53 impulses s^{-1}, $P < 0.05$). In the second group, six additional afferents (one Aδ-, $CV = 2.61$ m s^{-1}; five C-fibres, $CV = 1.03 \pm 0.37$ m s^{-1} responded consistently to 5 μg of U46619 following administration of the vehicle (2% $NaHCO_3$, Fig. 4*B*). The locations of the 12 afferent nerve endings are provided in Fig. 1.

Effect of blockade of TxA_2 receptors on activity of cardiac afferents during myocardial ischaemia

Representative tracings of a cardiac C-fibre afferent that responded to myocardial ischaemia in the absence and presence of BM13,177 are shown in Fig. 5. Ischaemia

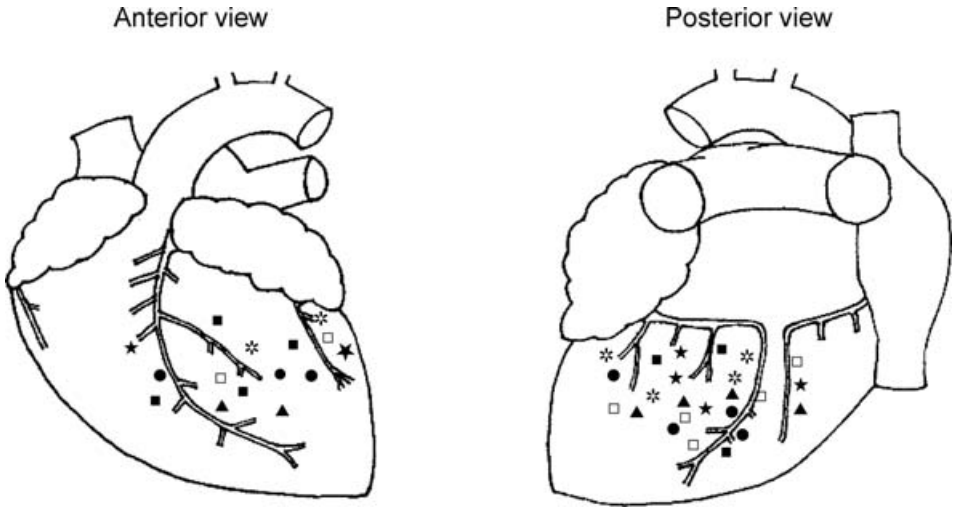

Figure 1. Location of the receptive fields of ischaemically sensitive cardiac afferents on epicardial surface of left ventricle
●, U46619 ($n = 7$); ▲, U46619 + BM13,177 ($n = 6$); ✲, repeat U46619 ($n = 6$); □, ischaemia + BM13,177 ($n = 7$); ■, repeat ischaemia ($n = 7$); ⋆, U46619 + PKC-(19–36) ($n = 6$).

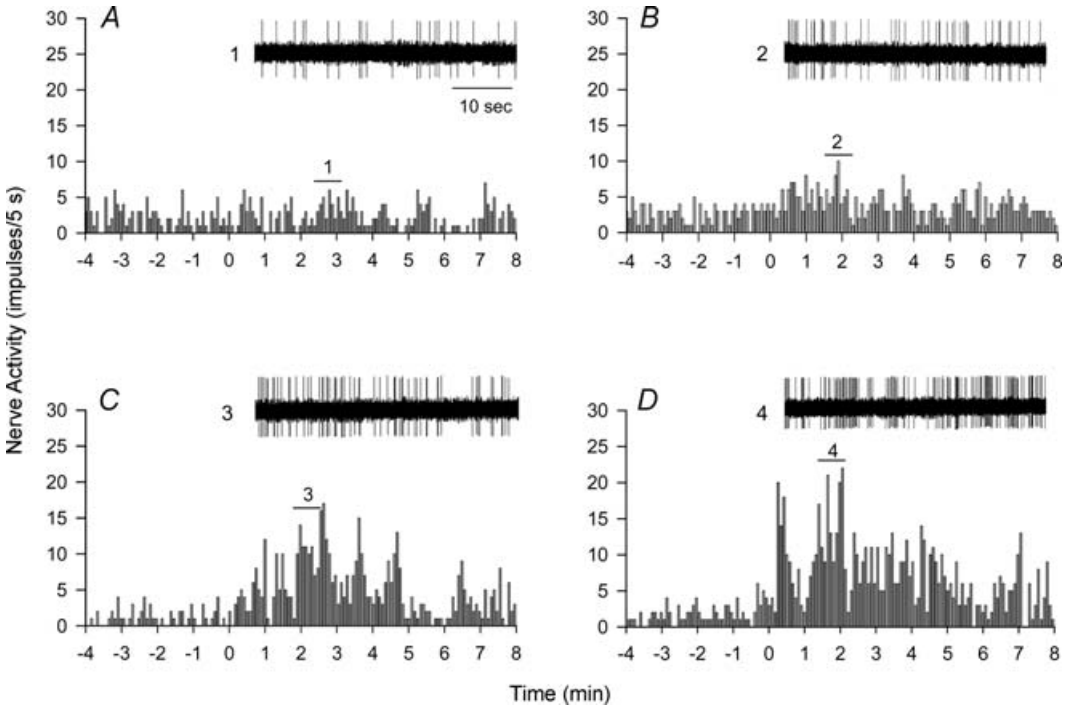

Figure 2. Representative tracings of ischaemically sensitive cardiac sympathetic afferent (conduction velocity (CV) = 0.42 m s^{-1}) innervating posterior wall of left ventricle
Myocardial ischaemia increased baseline activity from 0.78 to 2.35 impulses s^{-1}. Histograms *A–D* illustrate the responses of this afferent before and during injection of vehicle (2% ethanol, *A*), U46619 at 2.5 μg (*B*), 5 μg (*C*) and 10 μg (*D*) into the left atrium (LA). LA injection was conducted at 0 min. Neurograms 1–4 are representative tracings of discharge activity of the afferent, at times indicated by the lines above histograms.

increased discharge activity of this afferent from 1.23 to 4.47 impulses s^{-1} (Fig. 5*A*). Antagonism of TP receptors with BM13,177 (30 mg kg^{-1}, I.V.) attenuated by 48% the ischaemia-induced increase when averaged over a 5 min period (from 4.47 to 2.31 impulses s^{-1}) (Fig. 5*B*).

The responses of two groups of cardiac afferents to brief myocardial ischaemia are displayed in Fig. 6. In the first group, 5 min of ischaemia significantly increased the discharge activity of seven afferents (two Aδ-, CV = 3.43 and 3.65 m s^{-1}; five C-fibres, CV = 0.65 ± 0.26 m s^{-1}) from 0.75 ± 0.11 to 2.76 ± 0.56 impulses s^{-1} (Fig. 6*A*). After blockade of TP receptors with BM13,177, however, the ischaemia-induced increase in the activity of these cardiac afferents was significantly attenuated (2.76 ± 0.56 to 1.55 ± 0.27 impulses s^{-1}), compared with the initial period of ischaemia (Fig. 6*A*). In contrast, the responses of these afferents to BK application (3 μg, LA) were unaltered (0.72 ± 0.27 to 3.56 ± 0.38 impulses s^{-1} *versus* 0.74 ± 0.32 to 3.33 ± 0.52 impulses s^{-1}, $P > 0.05$) after BM13,177. In another group, seven additional afferents (one Aδ-, CV = 2.78 m s^{-1}; six C-fibres, CV = 0.78 ± 0.38 m s^{-1}) responded consistently to 5 min of repeated myocardial ischaemia in the presence of the vehicle (Fig. 6*B*). Locations of the 14 afferent nerve endings tested during ischaemia are provided in Fig. 1.

Figure 6*C* shows the summated 5 s nerve activity during 5 min of myocardial ischaemia in all seven cardiac afferents before and after administration of BM13,177. Similar to the changes in mean afferent nerve activity, the summated discharge activity during the entire 5 min period of

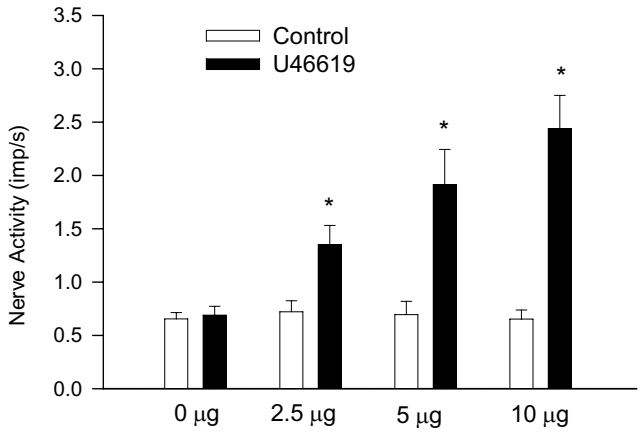

Figure 3. Bar graph showing peak impulse activity of seven cardiac sympathetic afferents before (open bar) and after (filled bar) left atrial injection of vehicle or U46619 (2.5–10 μg)
Columns and error bars represent means ± S.E.M. ($n = 7$). *$P < 0.05$ compared with control.

ischaemia (including both the early and late periods of stimulation) was attenuated by approximately 45% after blockade of TP receptors with BM13,177, indicating that BM13,177 blocks the early as well as the later afferent responses to myocardial ischaemia.

Influence of inhibition of PKC on responses of ischaemically sensitive cardiac afferents to U46619

The influence of PKC inhibition with the selective inhibitor PKC-(19–36) on the response of six ischaemically sensitive afferents (one Aδ-, CV = 2.58 m s^{-1}; five C-fibres, CV = 0.81 ± 0.23 m s^{-1}) to U46619 is shown in Fig. 7. The TxA_2 mimetic U46619 (5 μg, LA) stimulated all six cardiac afferents leading to a significant increase in discharge activity of these afferents (0.75 ± 0.24 to 2.74 ± 0.52 impulses s^{-1}). The responses of each of the afferents to U46619 were attenuated (2.74 ± 0.52 to 1.71 ± 0.23 impulses s^{-1}) by PKC-(19–36) (30 μg kg^{-1}, I.V.). PKC-(19–36) did not stimulate any of the six afferents. The locations of these afferents studied during inhibition of PKC are shown in Fig. 1. As mentioned above (Fig. 4*B*), afferents in the time control group responded consistently to repeated U46619.

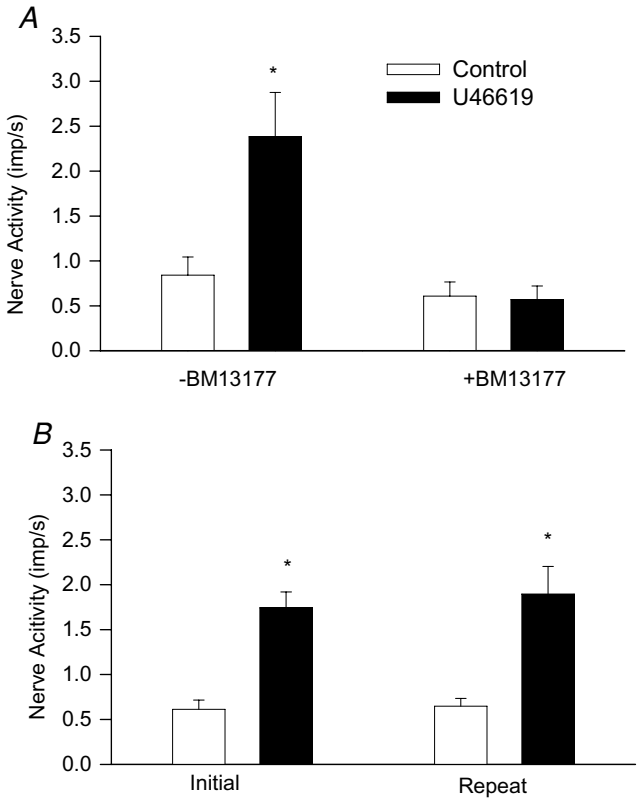

Figure 4. Responses of six cardiac sympathetic afferents to U46619 before and after treatment with BM13,177
A, response to U46619 before and after treatment with 30 mg kg^{-1} BM13,177, I.V. *B*, reproducibility of responses of six other cardiac sympathetic afferents to U46619. Columns and error bars represent means ± S.E.M. $^*P < 0.05$ compared with control; †$P < 0.05$ post-BM13,177 *versus* pre-BM13,177.

DiI labelling and immunohistochemical staining of TP receptors in DRG

Ten weeks following application of DiI into the pericardial space of two cats, DiI-labelled perikarya were observed bilaterally in the DRGs between T_1 and T_3, but not in L_1 ganglia of both animals. This pattern of labelling indicates that DiI was specifically transported from cardiac afferent nerve endings to cardiac afferent cell bodies located in the dorsal root ganglia between T_1 and T_3. In addition, we observed no DiI-labelled neurons in the DRGs between T_1 and T_3 in one cat that was not treated with DiI. TP-labelled receptors in neurons were noted in DRG sections obtained from both DiI-treated and -untreated cats. Importantly, we observed that, in DiI-treated animals, DiI-labelled DRG neurons were co-labelled with TP receptors in ganglia between T_1 and T_3. We counted a number of DRG sections containing neurons that were labelled with DiI, TP receptors or double-labelled with both DiI and TP receptors. We found an average of 79 ± 13 neurons labelled with DiI, 147 ± 13 neurons containing TP receptors and 65 ± 7 neurons double-labelled in each DRG section. Thus, 85% of DiI-labelled neurons contained TP receptors, while 45% of TP-labelled neurons co-labelled with DiI. Figure 8 shows examples of confocal images taken from a DRG section containing neurons double-labelled with DiI and TP receptors at T_1.

Discussion

Four new observations were made in this study. First, we found that injection of the stable TxA_2 mimetic U46619 into left atrium stimulated ischaemically sensitive cardiac spinal afferents in a dose-dependent manner. Second, administration of the TxA_2 receptor antagonist BM13,177 abolished the cardiac spinal afferent responses to U46619 and significantly attenuated the afferents' responses to regional myocardial ischaemia. Third, inhibition of PKC following administration of PKC-(19–36), a selective inhibitor of protein kinase C, attenuated the responses of ischaemically sensitive cardiac afferents to U46619. Finally, we found that TxA_2 receptors are expressed in cell bodies of cardiac sensory neurons located in the DRG of cats, assessed by double-labelling with the retrogradely transported DiI and TP receptors. Taken together, these data indicate that endogenous TxA_2 contributes to the activation of ischaemically sensitive cardiac spinal afferents during myocardial ischaemia through direct stimulation of TxA_2 receptors and that TxA_2 stimulates these cardiac

afferents, at least in part, through an intracellular PKC pathway.

TxA_2, formed from cyclic endoperoxides by the action of cyclooxygenase (COX) on arachidonic acid, is released from activated platelets during clinical myocardial ischaemic conditions, including both unstable angina and myocardial infarction as well as during provoked myocardial ischaemia in experimental animals. In this respect, a number of studies have demonstrated that large increases in TxA_2 coincide with episodes of myocardial ischaemia occurring in patients with unstable angina and myocardial infarction (Fitzgerald *et al.* 1986). Mehta and colleagues (Mehta *et al.* 1984*a*) observed that TxA_2 is increased in coronary venous plasma during spontaneous angina and pacing-induced angina in patients with coronary artery disease. Experimental evidence has also documented increases in transcardiac TxA_2 during induced ischaemia in animals with coronary artery stenosis (Folts *et al.* 1976; Lewy *et al.* 1980; Hirsh *et al.* 1981; Folts, 1994). Parratt & Cokerm (1981) reported an increase in TxA_2 concentrations in coronary venous plasma draining from the ischaemic region at least as early as 3 min after occlusion of a coronary artery branch. Thus, myocardial ischaemia increases the regional concentration of TxA_2, which then is available as a chemical stimulus of cardiac spinal afferents during ischaemia.

Previous studies have investigated the role of TxA_2 on vascular smooth muscle constriction and platelet activation (Arita *et al.* 1989; Furci *et al.* 1991). In contrast, the role of TxA_2 on the sensory nerve system remains poorly studied. Relatively few studies have evaluated the influence of TxA_2 on peripheral sensory nerves. Exogenous TxA_2 appears to be capable of stimulating hindlimb group III and IV somatic afferent nerves (Kenagy *et al.* 1997). Also, U46619 inhibits the knee-jerk reflex through a vagal pathway originating in the lung (Pickar, 1998). U46619 additionally can evoke tachypnoea and a depressor reflex response including bradycardia and hypotension through stimulation of the vagus nerve (Carrithers *et al.* 1994; Wacker *et al.* 2002). Infusion of U46619 into the inferior vena cava of cats likewise causes vagally mediated rapid shallow breathing and airway hyperresponsiveness (Aizawa & Hirose, 1988; Shams & Scheid, 1990; Karla *et al.* 1992). Finally, epicardial application of exogenous TxA_2 is capable of stimulating cardiac vagal chemosensitive afferents in rats (Sun *et al.* 2001). It is important to note that

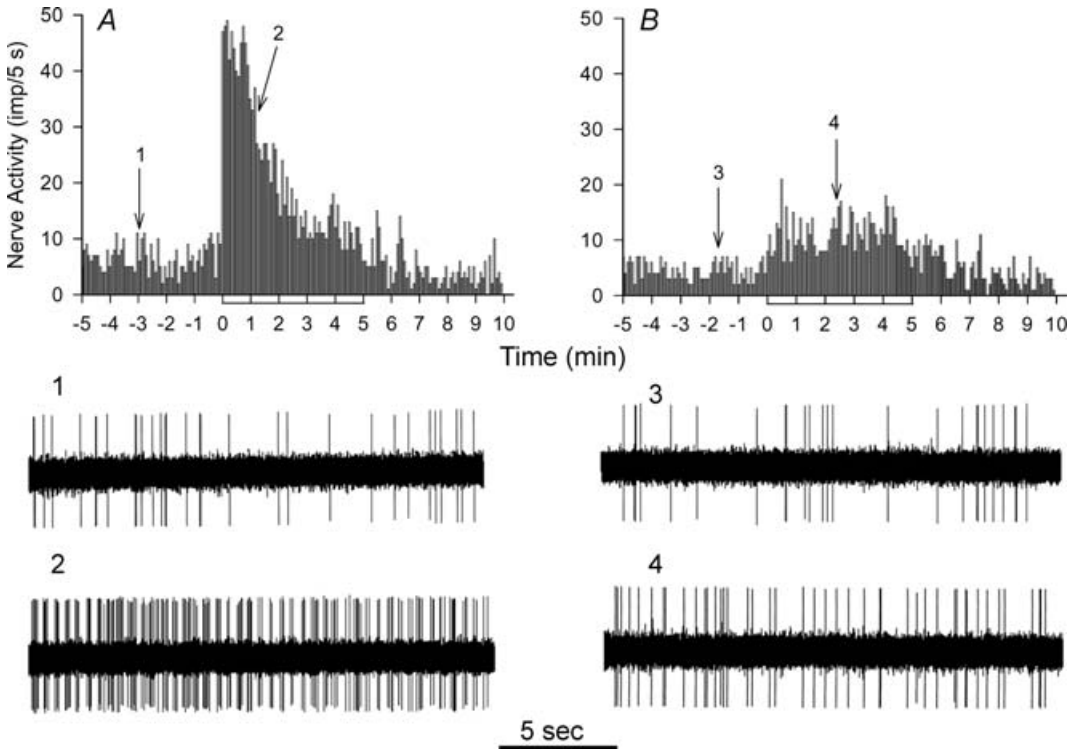

Figure 5. Neurohistogram showing response of a cardiac sympathetic C-fibre (CV = 0.33 m s^{-1}) innervating posterior wall of left ventricle to 5 min of myocardial ischaemia before and after treatment with BM13,177 (30 mg kg^{-1}, I.V.)

A, 5 min of myocardial ischaemia increased baseline activity of this afferent from 1.23 to 4.47 impulses s^{-1}. *B*, BM13,177 attenuated the increase (0.99–2.31 impulses s^{-1}) in discharge activity of this afferent during repeat ischaemia. Panels 1–4 are representative tracings of the discharge activity of the afferent at times indicated by the arrows above histograms.

none of these studies has examined the role of endogenous TxA_2. Furthermore, no previous study has evaluated the role of TxA_2 in the activation of sensory nerves during myocardial ischaemia.

We have shown previously that activated platelets stimulate cardiac spinal afferents (Fu & Longhurst, 2002*b*). TxA_2 is an important mediator released from activated platelets during ischaemia (Fitzgerald *et al.* 1986). However, it has been unclear whether sufficient endogenous TxA_2 is released to stimulate cardiac afferents during ischaemia. In the current study, we observed that BM13,177 abolished the responses of ischaemically sensitive cardiac spinal afferents to U46619 and attenuated the responses of afferents to myocardial ischaemia. Since BM13,177 is a specific TxA_2 receptor antagonist, which selectively blocks TxA_2 receptors but not the cyclooxygenase system including prostacyclin synthase and thromboxane synthase (Stegmeier *et al.* 1984; oude Egbrink *et al.* 1993), the present study provides the first evidence that endogenous TxA_2 is capable of stimulating cardiac afferents during ischaemia through the activation of TP receptors.

In addition to the well-known presence of TP receptors on platelets and smooth muscle cells (Coleman *et al.* 1994), recent evidence suggests the existence of TxA_2 receptors on glial cells in the central and peripheral nervous systems. In this respect, immunohistochemical studies have revealed the presence of TP receptors in oligodendrocytes and astrocytes associated with myelinated fibre tracts, most notably in the striatum, spinal cord and optic tract (Borg *et al.* 1994; Blackman *et al.* 1998). In addition, stimulation of TxA_2 receptors in the brain stem of rats by intracerebroventricular injection of U46619 elevates arterial blood pressure (Gao *et al.* 1997) suggesting that TP receptors may be associated with neurons, although indirect effects stemming from activation of nearby cellular elements like glia cannot be dismissed. With respect to its peripheral neural elements, Muja and colleagues (Muja *et al.* 2001) reported that TxA_2 receptors are located on Schwann cells in rat sciatic nerves.

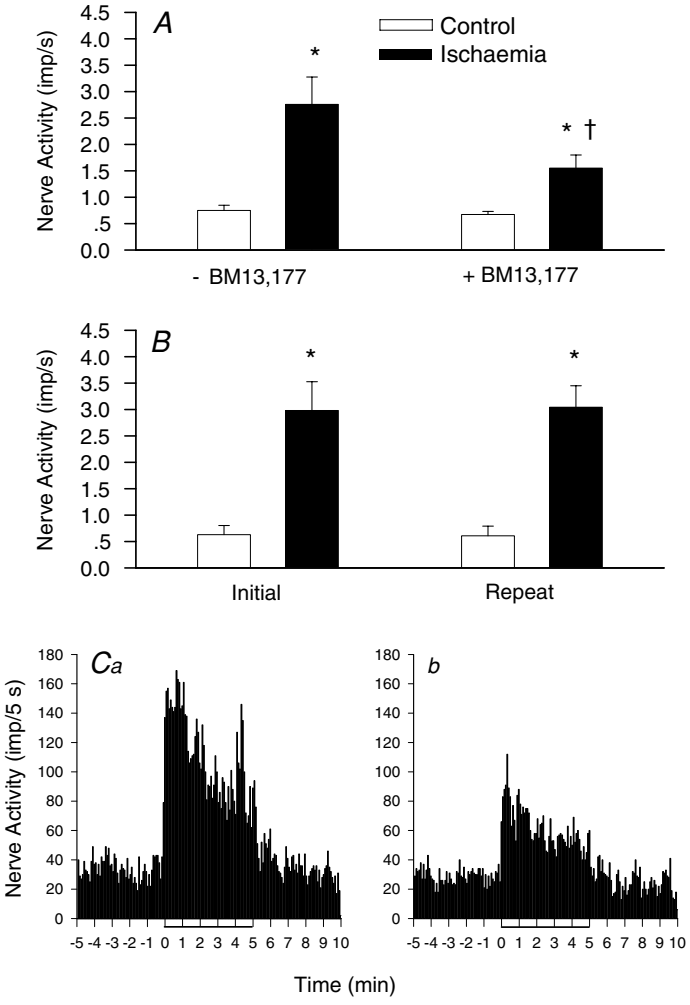

Figure 6. Bar graph summarizing changes in activity of seven cardiac sympathetic afferents before (open bar) and during (filled bar) 5 min of repeated myocardial ischaemia before and after treatment with BM13,177 (30 mg kg^{-1}, I.V.; *A*)

B shows consistent responses of seven other cardiac sympathetic afferents to myocardial ischaemia with vehicle (2% $NaHCO_3$) administered between first and second period of ischaemia. *C* provides neurohistograms of summated 5 s discharge activity from all seven cardiac afferents during ischaemia before (*a*) and after (*b*) treatment with BM13,177. Columns and error bars represent means ± S.E.M. *$P < 0.05$ compared with control. †$P < 0.05$ post-BM13,177 *versus* pre-BM13,177.

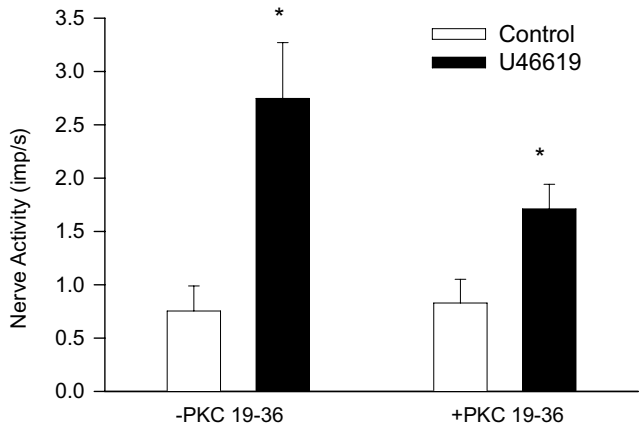

Figure 7. Responses of six cardiac sympathetic afferents to U46619 before and after treatment with PKC inhibitor, PKC-(19–36) (30 μg kg^{-1}, I.V.)
Columns and error bars represent means ± S.E.M. *$P < 0.05$ compared with control; †$P < 0.05$ post-PKC-(19–36) *versus* pre-PKC-(19–36).

Furthermore, TP receptor mRNA has been detected in nodose ganglion neurons (Wacker *et al.* 2005) although the organ(s) innervated specifically by these neurons was/were not identified. Our cardiac sympathetic afferent recordings suggest that TxA_2 might stimulate cardiac sympathetic afferents directly through activation of TP receptors located on the afferent endings, an assumption that was confirmed by our immunohistochemical data showing that the TP receptors are expressed in cardiac spinal afferent neurons in DRG. Data showing that vanilloid receptors are located on both DRG and cardiac spinal sensory nerve endings (Zahner *et al.* 2003) and on the nodose ganglia and vagal afferent nerve endings (Patterson *et al.* 2003) indicate that receptors found on neuronal cell bodies are also located on nerve endings.

TxA_2 potentially activates cardiac sensory nerves either through its direct action on afferent endings or by inducing the release of other endogenous mediators, which, in turn, stimulate sensory nerves. In this latter regard, our previous

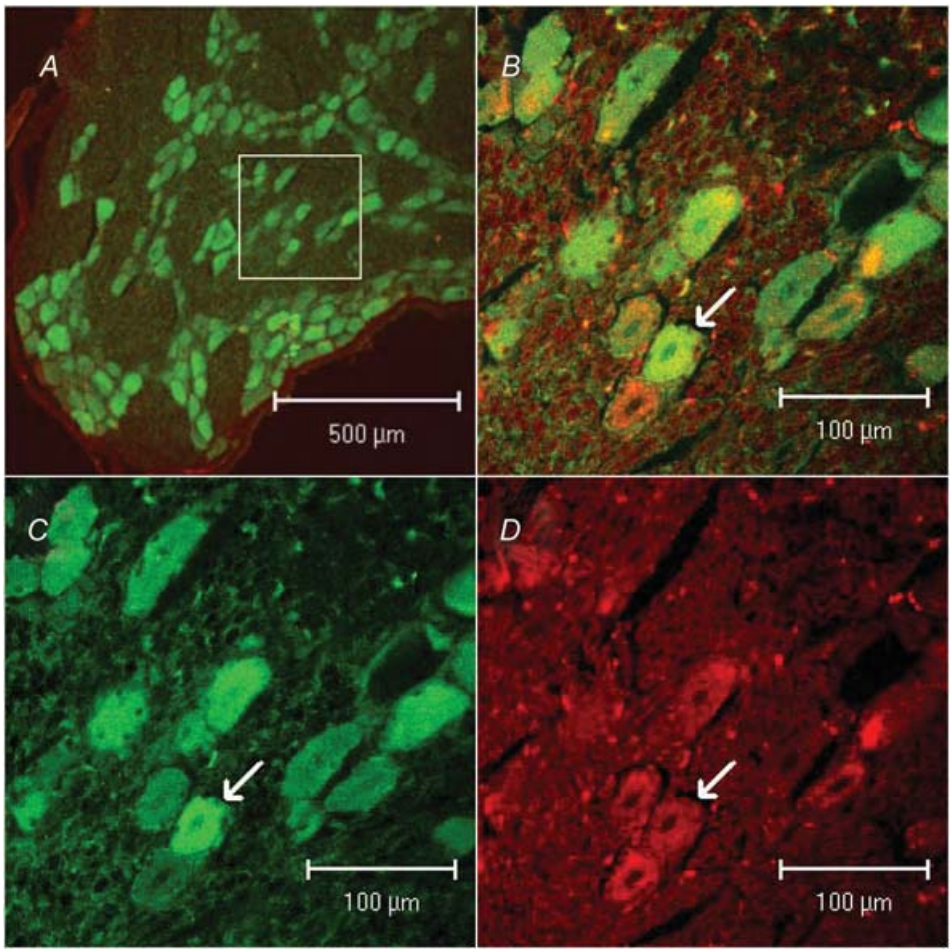

Figure 8. Confocal microscopic images of dorsal root ganglion (DRG) neurons double-labelled with DiI and TP receptors at spinal T_2 level in a cat
A, low-power photomicrograph; *B*, magnified region shown within box in *A*. Arrow indicates an example of co-localization of a DiI-labelled neuron with TP receptors. *B* is a merged image from *C* and *D*. Arrows in *C* and *D* indicate a neuron labelled with DiI or TP receptors, respectively. Scale bars in *A* and *B–D* represent 500 and 100 μm.

studies have documented that TxA_2 activates platelets to release serotonin and histamine to stimulate cardiac afferents (Fu & Longhurst, 2002*a*; Fu *et al.* 2005). However, in a recent preliminary study examining the reflex effects of TxA_2, we observed that neither histamine nor 5-HT_3 account for TxA_2's sympathoexcitatory reflex action (Phan *et al.* 2002; authors' unpublished observations). Additionally, in the current study we observed that early activation of cardiac afferents during ischaemia was inhibited by treatment with BM13,177 (30 mg kg^{-1}, I.V.), suggesting an early role for TxA_2 participating in the activation of cardiac afferents during ischaemia. There are several lines of evidence to support this conclusion. First, previous studies have demonstrated that BM13,177 at doses from 5 to 30 mg kg^{-1} (I.V.) selectively blocks TP receptors and eliminates the actions of TxA_2 on platelets and smooth muscle (Nossaman *et al.* 1992; oude Egbrink *et al.* 1993). Our observation that blockade of TP receptors with BM13,177 (30 mg kg^{-1}, I.V.) does not alter the responses of cardiac afferents to bradykinin stimulation suggests specificity of TP receptor blockade with this antagonist. Second, during ischaemia TxA_2 is probably released quickly from platelets since blockade of platelet GP IIb–IIIa receptors with tirofiban also attenuates the early activation of afferents (Fitzgerald *et al.* 1986; Fu & Longhurst, 2002*a*). Finally, cardiac spinal afferent nerve endings are located between myocytes and in close proximity to vessels where the TxA_2 is produced (Baker *et al.* 1980; Zahner *et al.* 2003). Taking these data together, we believe that during ischaemia TxA_2 stimulates cardiac afferents very quickly by activating TP receptors that are located directly on cardiac sensory nerve endings rather than by releasing other mediators from platelets to indirectly activate these afferent endings.

TxA_2 signals cellular events through a G protein-coupled receptor termed the TP receptor, primarily coupled to the Gq-dependent activation of PLC, which, in turn, produces phosphoinositide (PI) to mobilize intracellular Ca^{2+} and diacylglycerol (DAG) that activates protein kinase C (PKC) (Bevan, 1996). Previous data have shown that PKC plays a pivotal role in processes underlying activation and sensitization of sensory neurons in mammals (Bevan, 1996; Guo *et al.* 1998). For example, our previous studies documented that histamine and BK activate sympathetic visceral afferents during ischaemia, in part, through a PKC mechanism (Guo *et al.* 1998, 1999; Fu *et al.* 2005). In concert with these findings, Ahlgren & Levine (1994) reported that PKC inhibition with PKC-(19–36) decreases C-fibre hyperexcitability and hyperalgesia in diabetic rats but that PKC-(19–36) does not influence PGE_2- and PGI_2-induced hyperalgesia, which both act through the cAMP–PKA messaging system. TxA_2 also appears to alter neural transmission in the hippocampus through a PKC mechanism (Hsu & Han, 1996). We therefore speculated that TxA_2 stimulates ischaemically sensitive cardiac sympathetic afferents through a PKC intracellular mechanism. Experimental evidence supports this hypothesis. In this regard, we demonstrated that inhibition of the PKC pathway with PKC-(19–36) attenuates the response of these cardiac afferents to U46619. Since PKC-(19–36) is a highly selective inhibitor of PKC, the current data suggest that TxA_2 stimulates ischaemically sensitive cardiac spinal afferents, at least in part, through an intracellular PLC–PKC pathway.

Concern might be raised that we did not exclude an indirect effect of TxA_2 that might occur as a result of the generation of ischaemic mediators and other prostaglandins produced secondarily through the local action of TxA_2 on vascular smooth muscle-induced vasoconstriction and ischaemia. Thus, U46619 might cause myocardial ischaemia, which through the production of chemical stimuli other than TxA_2 would then be available to stimulate cardiac afferents. However, previous studies (Nichols *et al.* 1988) have demonstrated that intracoronary administration of 0.3–1 μg of U46619 reduces coronary blood flow by only 2–5%, which is insufficient to induce myocardial ischaemia. In the present study we injected 2.5–10 μg of U46619 into the left atrium. By the time this dose of the thromboxane mimetic reached the coronary vasculature only a small portion (5%) of the injected U46619 would be delivered to the coronary circulation since 5% of cardiac output is distributed to the coronary circulation. Thus, only approximately 0.125–0.5 μg of U46619 reached the coronary circulation, an amount that would be insufficient to induce myocardial ischaemia. Second, other previous studies have shown that U46619 stimulates blood vessels to release prostacyclin (PGI_2; Mehta *et al.* 1984*b*; Jeremy *et al.* 1985). However, in the current study we found that blockade of TxA_2 receptors with the selective TxA_2 receptor antagonist BM13,177 abolished the responses of these afferents to U46619. If PGI_2 contributed significantly to these responses, blockade of TP receptors with BM13,177 would not have completely eliminated the response. Furthermore, we have shown that TxA_2 receptors are located on perikarya of cardiac afferent neurons, suggesting that TxA_2 has the potential to be able to directly stimulate sympathetic afferents endings. As such, we believe that TxA_2 most likely directly stimulates cardiac afferents through a TxA_2 receptor mechanism that does not involve other mediators, such as prostaglandins like PGI_2.

In conclusion, using a combined neurophysiological and immunohistochemical approach, the present study demonstrates for the first time that endogenous TxA_2 is capable of directly stimulating ischaemically sensitive cardiac spinal afferents through the activation of TP receptors located on cardiac spinal sensory nerves. The action of TxA_2 on the cardiac afferents is mediated by TP receptors coupled to the PLC–PKC intracellular pathway. In a recent preliminary study we have observed

that endogenous TxA_2 can lead to excitatory cardiovascular reflex responses through the activation of these cardiac spinal afferents during ischaemia (Phan *et al.* 2002). These two studies reinforce the conclusion that this endoperoxide chemical signal serves an important role during ischaemia in activation of cardiac sensory nerves, which transmit information to the CNS ultimately leading to pain and sympathoexcitation. This information extends our understanding of the role of endogenous TxA_2 in pathophysiological responses to myocardial ischaemia. In view of these new findings, strategies to treat angina pectoris that incorporate blockade of TP receptors with selective TP receptor antagonists may be considered by physicians in the future. This therapeutic option could potentially relieve cardiac pain and the associated sympathoexcitatory reflex responses that can become exaggerated in some circumstances.

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